THE ROLE OF WNT5A IN MELANOMA IMMUNOPHENOTYPE REGULATION

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ABSTRACT

Melanoma is one of the most common cancers in the United States and worldwide. The incidence rates of melanoma continue to rise rapidly, especially over the past few decades. Although targeted therapies and immunotherapy have shown promise in the management of advanced melanoma, the acquired resistance in some patients emerges as a major hurdle within the clinic. Wnt5A has been identified as a potential molecular target due to its important role in melanoma progression and therapeutic resistance. It is believed that Wnt5A can regulate the secretion of inflammatory cytokines and chemokines that are involved in leukocyte trafficking. The mechanisms underlying the establishment of immune resistance by Wnt5A in the context of melanoma are not well understood. Here, we demonstrated that melanoma-derived Wnt5A downregulates the expression of inflammatory chemokines: CXCL10, which mediates immune responses by recruiting lymphocytes through the CXCR3 receptor; and CCL2, which binds to CCR2 and drives chemotaxis of monocytes. Levels of inflammatory cytokines, IFN-γ and TNF-α, were also reduced by Wnt5A, creating a less immunogenic microenvironment. The treatment of melanoma cells with recombinant Wnt5A reduced infiltration of macrophages and dendritic cells into the primary tumor site, owing to the reduced CCL2/CCR2 signaling. Finally, our immune profiling analysis via flow cytometry provided us with an optimized method of thoroughly investigating the effects of cancer-derived Wnt5A in the infiltration of immune populations. Together with the expression patterns of chemokine receptors: CXCR3 and CCR2 on the identified leukocytes, it would profoundly impact our future studies on

Wnt5A-mediated chemokine signaling and leukocyte trafficking that influence the tumor microenvironment. Overall, our work supports the negative role of Wnt5A in immunomodulation through downregulating inflammatory cytokine and chemokine expression and subsequent immune infiltration to the tumor microenvironment. Inhibition of Wnt5A could be used in combinatorial approaches with immunotherapy to treat advanced melanoma.

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ABBREVIATIONS

RGP	radial growth phase
VGP	vertical growth phase
TNM	tumor, nodes and metastasis
BRAFi	BRAF inhibitor
MAPK	motigen-activated protein kinase
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
CTLA4	cytotoxic T-lymphocyte-associated protein 4
ICIs	immune checkpoint inhibitors
WNT	Wingless/integrase 1
WNT5A	Wnt Family Member 5A
rWnt5A	recombinant Wnt5A
PCP	planar cell polarity
CE	convergent extension
ROR	the RAR-related orphan receptor
Ryk	the related to receptor tyrosine kinase
TME	tumor microenvironment
MDSCs	myeloid-Derived Suppressor Cells
MCP-1	monocyte attractant protein-1
CCL2	C-C Motif Chemokine Ligand 2
CCR2	C-C chemokine receptor type 2

CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C Motif Chemokine Receptor 3
TLR	Toll-like receptor
MyD88	Myeloid differentiation factor 88
NK cells	Natural Killer cells
YUMM	Yale University Mouse Melanoma
GEMM	genetically engineered mouse model
Pten	Phosphatase and tensin homolog
Cdkn2a	Cyclin Dependent Kinase Inhibitor 2A
MHC	major histocompatibility complex
APCs	antigen-presenting cells
BM	bone marrow
CCR7	C-C chemokine receptor type 7
CD86	Cluster of Differentiation 86
CD25	Interleukin-2 receptor alpha chain
CD62L	L-selectin
TCR	T cell receptor
Tcm cells	central memory T cells
Tem cells	effector memory T cells
Tregs	regulatory T cells
LPS	Lipopolysaccharides
MMPs	Matrix Metalloproteinases

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INTRODUCTION

Pathology of melanoma

Melanoma is a type of skin cancer that occurs when melanocytes, the melanin-producing cells, are mutated and become cancerous. Although invasive melanoma accounts for only about 1% of all skin cancers, due to the highly invasive nature of this disease, it is responsible for a large majority of skin cancer deaths[1,2]. Moreover, the incidence of melanoma has been rising rapidly over the past few decades. Particularly, the number of newly diagnosed invasive melanoma has increased by approximately 40% between 2009 and 2019[3]. The statistics raise awareness about melanoma as a severe public health concern.

Cutaneous melanoma progresses in a stepwise fashion. Primary melanoma develops from an initial radial growth phase (RGP) and may be followed by a vertical growth phase (VGP)[4,5]. Radial growth phase melanoma grows horizontally and radially within the epidermis or sometimes invades the dermis[4-6]. It is characterized by a flat and irregular lesion without the formation of a nodule[4-6]. In the vertical growth phase, the lesion grows vertically and deeper into the tissues, forming a tumor[4-6]. The VGP melanoma is invasive and has acquired competence of metastasis[4-6].

To describe the extent of cancer in clinics, growth phases are re-put to be sequential stages based on the TNM (tumor, nodes and metastasis) system. The staging process serves as an indicator of prognosis and can assist in treatment decisions. There are five stages of melanoma, as shown in **Figure 1**. The earliest stage of melanoma is stage 0, where the malignant tumor is *in situ* and confined to the

epidermis, the upper layer of the skin. Subsequently, the tumor invades from the epidermis into the dermis, known as stage I and stage II melanoma. Both stages are invasive but localized, with stage II melanoma penetrating deeper into the dermis and developing ulceration[1]. Stage III melanoma has spread into the regional lymph nodes or tissues and shown evidence of satellite or in-transit metastasis[1]. Stage IV melanoma is known as metastatic melanoma. The malignant tumor has traveled beyond the original tumor site and regional lymph nodes to more distant areas of the body, such as lungs, liver, brain, bones and intestines[1]. Melanoma can be treated most effectively in its early stages when localized (stage 0, I, and II). As indicated by 2017 AJCC Melanoma Staging and Classification, with surgical removal, individuals with Stage 0 melanoma have a ten-year overall survival rate of 99%-100%[7]. On the other hand, the ten-year survival rate for Stage IV melanoma patients was about 10% to 15%[7].

Therapeutic resistance in melanoma patients

Clinical breakthroughs of molecular-targeted therapy and immunotherapy have improved the prognosis for patients with invasive melanoma. Small molecules such as Vemurafenib and Dabrafenib have been designed to specifically inhibit the mutated $BRAF^{V600E}$ in the MAPK pathway, which is mutated in more than two-thirds of melanoma cases[8,9]. These BRAF inhibitors (BRAFis) have achieved benefits in both tumor burden and overall survival in metastatic melanoma patients[10-12]. However, the duration of response is limited by resistance to BRAFis and subsequent relapse[13]. Although monoclonal antibodies against immune checkpoint inhibitors (ICIs), such as anti-PD-1 (Pembrolizumab, Nivolumab) and anti-CTLA4 (Ipilimumab), have

demonstrated clinical efficacy and durable responses in treating metastatic melanoma, a proportion of 40-60% of patients present acquired resistance and relapse within 2 years[14-17]. The resistance to multiple ICIs is largely attributed to the immunosuppressive tumor microenvironment and the absence of T cell infiltration, characterized as “cold tumors”[18]. On the other hand, the hot tumor microenvironment is T cell inflamed and presents higher response rates to immunotherapy[18,19]. Therapeutic approaches that could turn cold into hot tumors would be necessary to improve the response to immunotherapy. Therefore, it emphasizes an urgent need to identify new molecular targets and explore mechanisms involved in immunomodulatory pathways to treat advanced melanoma.

Wnt5A signaling and its potential role in immunomodulation

Recent studies have reported that Wnt5A was overexpressed in BRAFi-resistant cell lines and patient tumors[13]. Wnt5A is a member of the Wingless/integrase 1 (WNT) family of lipid-modified glycoproteins that are involved in cell growth, motility, adhesion, polarity and differentiation during embryonic development[20,21]. Wnt signals are divided into two major categories: the canonical pathway, which relies on the accumulation and transcriptional activity of β-catenin, and the non-canonical pathway that is independent of β-catenin[21,22]. Wnt5A is a highly conserved non-canonical Wnt ligand regulating planar cell polarity (PCP), convergent extension (CE), and epithelial-mesenchymal interaction during embryonic morphogenesis by binding to the Frizzled-, ROR- and Ryk family receptors and activating the downstream PCP or Wnt/Ca^{2+} pathway[20,22]. In

addition to its critical role in embryogenesis, the upregulation of Wnt5A is emerging as an important event in cancer progression. Studies have demonstrated the involvement of Wnt5A in regulating a diverse array of tumor cell behaviors, including inflammation, migration and metastasis **(Figure 2)**[22]. In this work, we focused on the role of Wnt5A in tumor microenvironment and inflammation.

Cancer cells in the tumor microenvironment (TME) release cytokines and chemokines to regulate inflammation and dictate the shifting of immune cells to favor cancer progression[20]. Although Wnt5A is reported to regulate the inflammatory process in both infectious and inflammatory diseases, its role in either inflammation in the context of melanoma or tumor evasion from immune destruction has been poorly investigated. In 2021, Douglass et al. suggested that Myeloid-Derived Suppressor Cells (MDSCs) act as a major source of Wnt5A and also depend on Wnt5A to create an immunosuppressive microenvironment[3]. Therefore, we decided to expand on this data and explore the role of Wnt5A from melanoma cells in the immunomodulation of the TME. It is believed that Wnt5A mediates the production of inflammatory cytokines and chemokines to reshape the TME[23]. Given the well-established role of the majority of cytokines and chemokines in leukocyte migration during inflammation and immune surveillance, it is important to understand how cytokine and chemokine expression are influenced by Wnt5A. The identification of these cytokines and chemokines offers not only an insight into the immunomodulatory functions of Wnt5A but also a potential therapeutic target in treating melanoma.

The role of CCL2/CCR2 and CXCL10/CXCR3 signaling in leukocyte trafficking

Given the role of Wnt5A in regulating the suppressive activity of MDSCs[24], we queried whether Wnt5A from cancer cells has effects on chemokines involved in myeloid cell chemotaxis. CCL2 or monocyte attractant protein-1 (MCP-1) is a member of the CC chemokine family and binds to CCR2, a seven-transmembrane G-protein-coupled receptor. CCL2 signals act as a chemoattractant for myeloid and lymphoid cells, including monocytes[25], dendritic cells[26,27], macrophages[27], MDSCs[28], T cells[29], and NK cells[30]. Since CCL2/CCR2 signaling is critical in myeloid cell infiltration, it is of utmost importance to better understand the role of Wnt5A in this process.

Furthermore, as we discussed before, cold tumors that are limited in T cell infiltration exist as a major challenge for immunotherapy. The conversion of a “cold” into an inflamed “hot” tumor would require prior therapies to induce tumor T cell infiltration. Thus, we wonder whether Wnt5A is involved in the regulation of chemokine signaling that is responsible for T cell recruitment. CXCR3 is a seven transmembrane G protein-coupled receptor predominantly expressed on T lymphocytes[31-33]. CXCL10, known as interferon-γ inducible protein 10, is a pro-inflammatory chemokine that specifically activates the CXCR3 receptor. CXCL10/CXCR3 signaling exerts the “homing” function to $CXCR3^+$ T cells by attracting them towards inflammatory, infectious and neoplastic sites[34]. Therefore, investigations into the role of CXCL10/CXCR3 signaling in the Wnt5A-overexpressed tumor microenvironment would provide a deep understanding of tumor T cell infiltration and better treatments of cold tumors.

YUMM cell lines

To study the effect of Wnt5A on chemokine secretions, we utilized is Yale University Mouse Melanoma (YUMM) cell lines developed by Dr. Marcus Bosenberg. Murine cancer cell lines have been widely used in cancer research. The initial B16 mouse melanoma cell line was generated from a naturally arising melanocytic tumor in an inbred C57Bl/6 mouse[35]. Although the B16 cell line has been a powerful tool to study cancer metastasis, the driver mutations of B16 have not been well-defined and tumor immune responses are compromised due to low levels of MHC class I molecules[36,37] . Therefore, in order to make mouse models more experimentally tractable, immunocompetent GEMM (genetically engineered mouse model) with specific human-relevant oncogenic driver mutations are needed. The YUMM cell lines were established from melanocyte specific *Braf*/*Pten* mouse models.

In this project, the cell line we used, YUMM1.7, was derived from mice bearing a $BRAF^{V600E}$ mutation and *Pten* and *Cdkn2a* inactivation[36]. The YUMM cell lines are generally recognized as poorly immunogenic due to a low number of somatic genetic changes[38,39]. An exception to this is YUMM2.1 cell line. When implanted into mice and treated with anti-PD1 therapy, it induces abundant immune cell infiltrates and strong anti-tumor responses[40]. The robust immune response of YUMM2.1 melanoma is characterized by high levels of inflammatory and chemotaxis-related genes that are important in immune cell infiltration and T cell priming[39,41]. Therefore, YUMM2.1 cells may not be ideal for studying the innate and acquired resistance to immunotherapy. YUMM1.7 cells, as demonstrated by Kugel and Weeraratna et al. (2018), are useful to

study the age-related responses to PD-1 inhibition and aged-related changes in the immune microenvironment. In our study, we continued to use YUMM1.7 to explore the impact of Wnt5A on the tumor immune microenvironment.

Our hypothesis is that Wnt5A can downregulate the expression of pro-inflammatory chemokines, such as CCL2 and CXCL10, and/or induce the overexpression of anti-inflammatory cytokines. The subsequent reduction in the recruitment of immune cells to the primary tumor shifts the immune microenvironment to favor melanoma progression. The mouse chemokine array and qPCR analysis on Wnt5A-overexpressed YUMM1.7 cells provided us with the expression patterns of inflammatory cytokines and chemokines. By performing flow cytometry on immune populations from the tumor and lymphoid organs, we further examined the immune profiles of transgenic mouse models as well as the influence of Wnt5A on chemokine signaling and immune infiltration.

MATERIALS AND METHODS

Cell Culture

YUMM1.7 melanoma cell lines were obtained from Dr. Marcus Bosenberg, Yale University. YUMM1.7 cells were maintained in Gibco™ DMEM/F-12 medium (Thermo Fisher Scientific), supplemented with 10% FCS, 1% penicillin and streptomycin, and 1% L-Glutamine. FS4, FS5, FS12 and FS14 melanoma cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, and 1% penicillin and streptomycin. 1205Lu cells and MW164 cells were cultured in a 4: 1 mixture of MCDB 153 medium (Sigma) with L-15 medium (Cellgro) supplemented with 2% FBS and 1.6 mM $CaCl_2$. All cell lines were cultured at 37°C in 5% CO_2 and the medium was replaced as required.

Lentiviral Transduction and Wnt5A Induction

The doxycycline-inducible Wnt5A vector: pLV[Exp]-mCherry:T2A:Bsd-TRE3G>hWNT5A was designed by Dr. Mitchell Fane and was sourced from vector builder **(Figure 3A)**. Lentiviral production (Lenti-X 293T) was performed by Dr. Mitchell Fane in accordance with the protocol provided by the Broad Institute. YUMM1.7 cells were transduced with lentivirus in the presence of 10 µg/ml polybrene (Sigma) for 48 hours and selected through puromycin and selection markers (mCherry). 1 µg/ml of doxycycline was used to induce the overexpression of Wnt5A.

Reverse Transcription

RNA from both Dox-induced and No dox-added groups was extracted using QIAGEN RNeasy Mini Kit (#74106) according to the manufacturer's protocol. The extracted RNA was quantified via NanoDrop spectrophotometer (Thermo Fisher

Scientific). 2 ng of RNA were reverse transcribed using Bio-Rad iScript cDNA Synthesis Kit (#1708891) following the manufacturer's instructions.

Real-time Quantitative PCR

Specific primers were customized and obtained from Integrated DNA Technologies. The primer sequences have been described in **Table 1**. qPCR was performed with Fast 96-well and SYBRgreen (Thermo Fisher Scientific) on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method and Student *t* test.

Table 1 Primer sequences designed for genes of interest.

Gene	Sense	Anti-sense
Beta-Actin	gaggtatcctgaccctgaagta	cacacgcagctcattgtaga
Wnt5A	attcttggtggtcgctaggta	gcgcttctccgatgtactgc
CCL2	cactcacctgctgctactca	gcttggtgacaaaaactacagc
CCL5	tttgcctacctctccctcg	cgactgcaagattggagcact
CXCL1	gctgggattcacctcaagaa	tggctatgacttcggtttgg
CXCL9	agagtagggaccacagactatt	gcctttctacctctcacacatac
CXCL10	tcaggctcgtcagttctaagt	ccttgggaagatggtggttaag
CXCL11	aagcaagctcgcctcataat	tcctggcacagagttcttattg
CXCL12	ggagccatagtaatgccagtag	ctgacaccggaaagctacaa
IFNG	acagcaaggcgaaaaaggat	tgagctcattgaatgcttgg
TNFA	ccaccacgctcttctgtctac	agggtctgggccatagaact
IL-6	gacaaagccagagtccttcagagag	ctaggtttgccgagtagatctc
IL-10	cttactgactggcatgaggatca	gcagctctaggagcatgtgg

Proteome Profiler Array

YUMM1.7 cell lines were plated and lysed with RIPA lysis buffer supplemented with proteinase and phosphatase inhibitors. The whole protein extract was quantified via a Pierce™ BCA protein assay kit (#23225, Thermo Fisher Scientific). The Proteome Profiler Mouse Chemokine Array (#ARY020, R&D Systems) was used to analyze the

expression profiles of chemokines in 200 µg of cell lysates in accordance with the manufacturer's protocol. The array membranes were exposed to X-ray film for multiple exposure times and images were captured by Syngene G:Box. Densitometry analysis was performed by Image J.

Western Blotting

Total protein lysate was quantified by procedures mentioned above and 50 µg was prepared and loaded into precast NuPAGE™ 4-12% Bis-Tris Protein Gels (#NP0321BOX, Thermo Fisher Scientific). Gels were run at 160 volts. Samples were transferred onto PVDF membrane via the iBlot system (Invitrogen). The membranes were then blocked with 5% milk in TBST for 1 hour and incubated with the primary antibody diluted in 1% BSA/TBST at 4 °C overnight. The membranes were washed in TBST. 0.2 µg/ml of the corresponding HRP conjugated secondary antibody was applied to the membranes and incubated at room temperature for 2 hours. Proteins were visualized by ECL prime and detected by ImageQuant™ LAS 4000 (GE Healthcare Life Sciences).

Wound healing assay

YUMM1.7 cells were seeded in a 6-well plate to reach 80% confluency. Cells were treated with 1 ng/ml of doxycycline and then starved with DMEM/F12 medium overnight. The wound was created by scratching horizontally with a 200 ul sterile pipette tip. After scratching, cells were maintained in DMEM/F-12 medium, supplemented with 10% FCS, 1% L-Glutamine, 1% penicillin and streptomycin. The wounds were photographed at the beginning (0h) under the microscope using the 4x objective lens.

The dishes were taken out of the incubator to be examined periodically and then returned to resume incubation.

Immunohistochemistry Assay

Primary YUMM1.7 melanoma tumors were sectioned and embedded in paraffin. Tumor sections were deparaffinized using xylene and subsequently rehydrated by a series of different concentrations of ethanol. Heat-mediated antigen retrieval was conducted by treating sections with antigen retrieval buffer (#H-3300, Vector Laboratory) for 20 minutes. Tumor sections were blocked with hydrogen peroxide blocking reagent (#ab64218, Abcam) for 15 minutes, which was followed by protein block (#ab64226, Abcam) for 30 minutes. Antibodies of interest (Wnt5A, #mab645, R&D systems) were prepared with antibody diluent (#S0809, Dako) and applied to slides and incubated at 4°C overnight in a humidified chamber. Samples were washed with PBS and incubated with biotinylated goat anti-rabbit secondary antibody (#ab64256, Abcam) for 1 hour. Samples were washed with PBS and treated with Streptavidin-HRP for 30 minutes. Samples were washed with PBS and incubated with AEC (3-amino-9-ethyl-1-carbazole) chromogen (#1T0080T, Enquire Bioreagents) for a certain amount of time and counterstained with Mayer's hematoxylin (Millipore Sigma) for 10 seconds. The samples were rinsed with PBS and mounted in Aquamount (#H-5501-60, Vector Laboratory).

Tissue processing and Flow Cytometry

Flow cytometry was carried out using a Northern Lights Flow Cytometer (Cytek Biosciences). Prior experiments with tumors were performed by Dr. Stephen Douglass

and analyzed by me under his guidance. Isolation of leukocytes from spleens and bone marrow was performed in assistance with Dr. Stephen Douglass. Tumors no larger than 1500 mm^3 were isolated and chopped into small fragments and incubated in the Tumor Dissociation Kit, mouse (#130-096-730, Miltenyi Biotec) at 37 °C for 1 hour. Isolated tumors and spleens were mashed through a 4 µm cell strainer. Bone marrow was harvested by stripping the leg of skin and muscle and isolating the femur of mice and flushing out bone marrow from the center of the bone using a 25G needle. Harvested tumor and spleen cells bone marrows were centrifuged to remove the supernatant and resuspended with ammonium chloride (ACK) RBC lysis buffer for 1 minute. 4 ml of FACS buffer (PBS with 1% FCS and 5mM EDTA) were added and centrifuged to discard the supernatant. The resulting cell pellet was resuspended and cells were rinsed and stained with antibodies in MACS buffer (0.5% FCS and 2.5mM EDTA in PBS) for 1 hour at 37°C in the dark. The antibodies used are listed in **Table 2, Table 3** and **Table 4**.

Cell surface staining:

Antibody combinations mentioned in **Tables 2** and **3** were in 1:200 dilutions. Single staining (for each fluorochrome) was used as a reference control. After staining, cells were washed with 1ml of FACS buffer and resuspended in 500µl of PBS.

Intracellular staining (Foxp3):

Following the completion of cell surface staining, cells were pelleted by centrifuging at 400 xG for 5mins. The pellet was resuspended in 1ml of prepared Fix/Perm buffer (x4, Biolegend) and left for 1hour at room temperature in the dark. 1ml

of Perm Buffer (x10) was added on top of the suspension and centrifuged at 400 xG for 5mins. The cell pellet was resuspended and incubated with 200 µl of Foxp3 antibody (listed in **Table 4**) diluted 1:200 in perm buffer for 1 hour in the dark. After staining, cells were washed with 2 ml of perm buffer and resuspended in 500 µl of PBS.

FACS analysis was performed using FlowJo software.

Statistical Analysis

All the data were repeated in duplicate at least. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical analysis was performed with GraphPad Prism software. Comparison between groups was conducted using Student two-tailed t-test to determine p values. P-value <0.05 was considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; N.S., not significant).

Table 2 Antibodies and fluorochromes used to stain myeloid cell population.

Myeloid Cell Populations				
Company	**Catalog #**	**Target**	**Fluorochrome**	**Clone**
BioLegend	103112	CD45	PerCP-Cy5.5	30-f11
Invitrogen	25-0112-81	CD11b	Bv510	M1/70
BioLegend	123117	F4/80	APC-Cy7	BM8
BD Bioscience	553104	Ly6C	AF700	AL-21
BD Bioscience	561104	Ly6G	Pe-Cy7	1A8
BioLegend	117339	CD11c	Bv650	N418
BioLegend	114406	MHCII	FITC	25-9-19
BioLegend	120119	CCR7	Bv421	4B12
BioLegend	150621	CCR2	Bv785	SA203G11
BioLegend	105007	CD86	PE	GL-1

Table 3 Antibodies and fluorochromes used to stain Lymphocytes.

Lymphocyte Populations				
Company	**Catalog #**	**Target**	**Fluorochrome**	**Clone**
BioLegend	103112	CD45	PerCP-Cy5.5	30-f11
BioLegend	100553	CD4	FITC	RM4-5
BioLegend	126609	CD8	PE	YTS156.7.7
BioLegend	137627	Nkp46	AF647	29A1.4
BioLegend	101915	CD25	Pe-Cy7	3C7
BioLegend	104453	CD62L	Bv650	MEL-14
BioLegend	109109	PD-1	APC-Cy7	RMP1-30
BioLegend	120119	CCR7	Bv421	4B12
BioLegend	150621	CCR2	Bv785	SA203G11
BioLegend	126527	CXCR3	Bv510	CXCR3-173

Table 4 Antibodies and fluorochromes used to stain Tregs.

Regulatory T cells				
BioLegend	103112	CD45	PerCP-Cy5.5	30-f11
BioLegend	100553	CD4	FITC	RM4-5
BioLegend	126410	Foxp3	AF647	MF-14

RESULTS

Induction of Wnt5A expression in YUMM1.7 cells

YUMM1.7 melanoma cells transduced with plasmid vectors were examined for Wnt5A expression using both western blotting and qPCR. The cells treated with doxycycline were referred to as the dox-treated group, and otherwise, it was named the no dox-treated group. In the absence of doxycycline, the expression of Wnt5A by YUMM1.7 cells was comparable to the previously characterized Wnt5A-low human melanoma cell lines (FS12, FS14 and WM164) (**Figure 3C**). After the treatment of doxycycline, Wnt5A expression was dramatically increased in YUMM1.7 cells (**Figure 3C**). It was quantified to be approximately 50 times more than the previously characterized Wnt5A-high melanoma cell lines (FS4, FS5 and 1205Lu). As indicated by **Figure 3B**, *Wnt5A* transcripts also showed more than an 80-fold increase in the dox-treated group. To further visualize Wnt5A expression patterns *in vivo*, dox-induced YUMM1.7 cells were injected sub-dermally into C57Bl/6 mice. Tumor sections in the dox-treated group showed high levels of Wnt5A compared to the no dox-treated group (**Figure 3D**). Interestingly, the no dox-treated group also expressed certain amounts of Wnt5A due to the endogenous expression in melanoma cells.

Melanoma-derived Wnt5A downregulates the expressions of inflammatory cytokines and chemokines: CXCL10, CCL2, IFN-γ and TNF-α

Since Wnt5A has been implicated as a regulator of inflammation, we wanted to assess whether Wnt5A can modulate the expression of chemokines by melanoma cells.

Protein extracts from the dox-treated and no dox-treated group were evaluated using a mouse chemokine array (**Figure 4B**). This analysis showed that Wnt5A downregulated the expression of several chemokines, including CXCL10, CCL2, CXCL1 and CCL5 (**Figure 4A**). To validate the effect of Wnt5A on chemokine reduction, we performed real-time qPCR analysis on several cytokine/chemokine transcripts. The levels of *CXCL10* and *CCL2* transcripts significantly decreased with the overexpression of Wnt5A (**Figure 4C**). Of note, CXCL9 and CXCL11, which share the same chemokine receptor CXCR3 with CXCL10, did not show a reduction in the transcription level in the dox-treated group (**Figure 5A**). Moreover, Wnt5A did not downregulate *CXCL1* and *CCL5* at the transcription level (**Figure 5A**), ruling out their participation in immune regulation in YUMM1.7 cells.

Previous studies have shown that the upregulated Wnt5A can induce the expression of IL-6 in immune and non-immune cells[42]. Besides promoting pro-inflammatory cytokine secretion, Wnt5A stimulates the secretion of anti-inflammatory cytokine IL-10 in immune cells through Wnt5a/TLR/MyD88 pathway[43]. Furthermore, TNF-α and IFN-γ play controversial roles in melanoma. In addition to anti-tumor effects, they have been demonstrated to enhance the expression of PD-L1 in melanoma, leading to adaptive immune resistance and melanoma relapse[44-46]. Therefore, we were interested to see whether the increased Wnt5A signaling can modulate the release of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-6) and anti-inflammatory cytokines (IL-10) in our case to trigger immunosuppression. As shown in **Figure 5B**, there were no significant changes in the transcription levels of *IL-6* and *IL-10* in the dox-treated group.

Therefore, Wnt5A is not associated with the regulation of these two cytokines in YUMM1.7 cells. Of note, the expressions of *IFNG* and *TNFA* were decreased with the overexpression of Wnt5A and differed significantly from the no dox-treated group (**Figure 5B**). If TNF-α and IFN-γ are directly involved in tumor promotion and immune resistance in YUMM1.7 melanoma, the expression levels should be increased. The downregulated expressions we observed instead cannot support the assumption of their direct contribution to immunosuppression. Interestingly, previous studies suggested the involvement of IFN-γ and TNF-α in macrophage polarization. The polarization to M1 or M2 macrophages and their migrations to the tumor microenvironment could be another important aspect to study the immunosuppression. The possible impact of IFN-γ and TNF-α on macrophage polarization and recruitment will be discussed later.

Enhanced invasive ability of Wnt5A-overexpressed YUMM1.7 cells

Weeraratna et al. have previously demonstrated that Wnt5A signaling can increase the invasive ability of melanoma cells[2]. To understand the impact of the downregulated cytokines and chemokines on YUMM1.7 cells, we tested cell motility *in vitro*. In the wound healing assay, we examined cell migration in response to the scratch wound in the absence or presence of doxycycline treatment. Images of scratch areas from the time points 0, 3, 5, 7, 9, 11 hours are illustrated in **Figure 5C**. When treated with doxycycline, the scratch was closed within 11 hours, whereas the no dox-treated group did not show apparent signs of the scratch closure. Therefore, the decreased

cytokine and chemokine signaling in the Wnt5A-overexpressed YUMM1.7 cells may correlate with the enhanced invasion.

Flow cytometric analysis of macrophages and dendritic cells from the tumor and spleen

To further visualize the effect of Wnt5A and chemokine signaling on the immune infiltration, YUMM1.7 cells were implanted into C57Bl/6 mice and treated with either 200 ng/ml rWnt5A (recombinant Wnt5A) or PBS every 3 to 4 days by Dr. Stephen Douglass. The tumor and spleen were isolated and single cells were analyzed by flow cytometry. Since CCL2 is a potent chemoattractant protein for monocytes[25,47], we were specifically interested in the infiltration patterns of these cells. Monocytes have a wide range of plasticity and depend on the TME to differentiate to macrophages and dendritic cells. There were significant decreases in the levels of macrophages and dendritic cells in the tumor with the stimulation of rWnt5A (**Figure 6**), which were not observed in the spleen, suggesting the Wnt5A-related reduction is tumor-specific. On the contrary, animals treated with rWnt5A had significantly increased the frequencies of macrophages and dendritic cells in the spleen (**Figures 6A&B**), probably due to the increased amounts of tumor antigen. Overall, Wnt5A can reduce the tumor-specific infiltration of macrophages and dendritic cells and potentially create an immunosuppressive microenvironment for cancer metastasis.

The receptor CXCR3, as introduced before, is predominantly expressed on T lymphocytes[31]. Its ligand, CXCL10, was demonstrated to be downregulated by cancer-

derived Wnt5A in our experiments. Therefore, we wondered whether the decreased CXCL10 expression would influence T cell recruitment. Interestingly, no significant changes in the infiltration of tumor $CD4^+$ and $CD8^+$ T cells were observed in the rWnt5A-treated group (**Figures 6E&F**). There were also no differences in the frequencies of spleen T cells with the stimulation of rWnt5A (**Figures 6E&F**). Overall, the decreased level of CXCL10 in the tumor microenvironment exerts a very limited impact on T cell recruitment.

Immunophenotyping of myeloid cell populations in murine bone marrow and spleen

Recent breakthroughs in tumor immunotherapy have been driven by a deeper understanding of immunoregulatory processes in the tumor microenvironment. We believe that continued analysis of basic details of the immunologic features in mouse models enables the improvement of current immunotherapeutic strategies and the identification of potential molecular targets. As discussed before, CCL2/CCR2 signaling acts as an important player in monocyte recruitment and CXCL10/CXCR3 signaling is predominantly involved in attracting T lymphocytes. Wnt5A downregulated CCL2 and CXCL10 at both the transcription and protein levels (**Figure 4**). Therefore, we wondered whether the overexpression of Wnt5A would influence the expressions of CCR2 and CXCR3 on immune populations and regulate their migrations into the tumor.

To characterize the murine immune cell populations, Dr. Stephen Douglass and I performed flow cytometry on leukocytes isolated from bone marrow and spleen. The

gating strategy developed by Dr. Stephen Douglass and myself is shown in **Figure 7**. After the debris was eliminated by FSC-A vs. SSC-A (data not shown) and single cells were selected by FSC-H vs. FSC-A gating (data not shown), immune cells were identified by the expression of CD45, previously known as CLA (common leukocyte antigen) (**Figures 7&8**). The percentages of immune cells in the bone marrow and spleen were comparable (**Figures 7&8**). Myeloid cells were then identified based on the surface marker CD11b. Myeloid cells accounted for the majority of immune cells in the bone marrow (87.7%) and spleen (75.3%) (**Figures 7&8**).

Within the myeloid compartment, Ly6C and F4/80 were used to define macrophages. Dendritic cells were identified based on the expression of surface CD11c. Neutrophils were distinguished by myeloid cell marker CD11b, then F4/80 negative populations were selected to rule out macrophages and then the neutrophil-specific marker Ly6G was used. As potent antigen-presenting cells (APCs), the activated neutrophils often differ from resting neutrophils based on the upregulation of MHC class II[48]. Therefore, the activated neutrophil populations were characterized by high levels of MHC class II. MDSCs were identified by the expression of myeloid cell marker CD11b and the lack of surface marker F4/80. Also, MDSCs were generally recognized as MHC class II negative[49]. Two populations of MDSCs, M-MDSCs and PMN-MDSCs, were distinguished by the proportion of the expression of surface markers: Ly6C and Ly6G. As shown in **Figures 7&8**, MDSCs towards the monocytic lineages (M-MDSCs) are $Ly6C^{hi}Ly6G^{-}$, while polymorphonuclear MDSCs (PMN-MDSCs) are $Ly6C^{lo}Ly6G^{+}$.

The observed frequencies of macrophages, dendritic cells and two populations of MDSCs in BM and spleen were similar (**Figures 7&8**). Notably, there were very few percentages of M-MDSCs in both bone marrow (2.92%) and spleen (1.51%). Considering that MDSCs accumulate in the tumor or chronically inflamed site to suppress immune cell effector functions, it is reasonable to observe low numbers of M-MDSCs in mice without pathology and tumor. Further experiments would be conducted on YUMM1.7 melanoma-bearing mice to examine the tumor-specific infiltration of M-MDSCs. Moreover, although bone marrow harbors substantial populations of immune cells, it accommodated a lower percentage of the activated neutrophils (11.5%) than the spleen (88.9%) (**Figures 7&8**). It suggests that there are considerable amounts of immature myeloid progenitors in the bone marrow. The acquisition of antigen-presenting functions in neutrophils and other antigen-presenting cells mainly happens in the spleen rather than the bone marrow.

Further characterization of macrophage and dendritic cell subsets was based on the activation marker CD86 and chemokine receptor CCR7. Macrophages are phagocytic cells that play an important role in innate immunity and activating adaptive immunity. There are two categories of macrophages: M1 and M2. M1 macrophages are classically activated and secrete pro-inflammatory cytokines to initiate host defense and anti-tumor properties[50]. Polarized M2 macrophages produce anti-inflammatory cytokines alternatively, which suppress the inflammatory response, therefore inhibiting tumor immunity and enhancing angiogenesis and ECM remodeling[50]. M1 macrophages were distinguished from M2 by high expression of MHC class II and CD86. CD86 is a co-

stimulatory molecule expressed on antigen-presenting cells (APC) such as macrophages and dendritic cells, which is required for T cell activation. Dendritic cells express minimal amounts of CD86 in the absence of antigen but upregulate the CD86 levels during antigen presentation[51,52]. As shown in **Figure 7**, bone marrow served as a reservoir for both M1 and M2 macrophages though M1 is dominant (69.3%). Spleen macrophages were mainly polarized into M1 state as there were 97.9% of M1 populations (**Figure 8**). As for dendritic cells, there was only 25.1% expressing high levels of MHC class II and CD86 in the bone marrow. The majority of spleen dendritic cells (94.5%) were activated and maturated upon the stimulation of antigen, which is reasonable due to the nature of spleen as an antigen-presenting site.

The interaction between chemokine receptor CCR7 and its ligands CCL9/CCL21 plays a critical role in regulating the specific chemotaxis of M1 macrophages to the injured or inflammatory tissue[53]. Additionally, CCR7 signaling is essential for the migration of dendritic cells from the affected tissue to lymphoid organs, which allows for the antigen presentation to cognate T cells and the activation of adaptive immunity[25,54]. In the spleen, a higher frequency (64.6%) of M1 macrophages with the expressions of MHC class II and CCR7 was observed compared to the bone marrow (13.5%). It is possible that significant amounts of M2 macrophages were derived from precursor cells in the bone marrow, which reduces the proportion of M1 populations. The frequencies of dendritic cells expressing both MHC class II and CCR7 in the bone marrow and spleen were comparable (**Figures 7&8**).

Immunophenotyping of lymphocyte populations in murine bone marrow and spleen

Different subsets of T lymphocytes in bone marrow, spleen, and blood were also characterized by flow cytometric analysis. $CD4^+$ and $CD8^+$ T lymphocytes were identified by their surface markers: CD4 and CD8, respectively. Markers consistent with T cell activation such as CD25 and PD-1 and the marker that distinguishes central memory T cells, CD62L, were used to characterize T cell subsets in the bone marrow and spleen. CD25 is the alpha chain of the interleukin-2 (IL-2) receptor. IL-2/CD25 signaling is required for T cell development and antigen-specific T cell responses[55]. Upon activation by T cell receptor (TCR) and co-stimulatory molecules, T cells upregulate the expression of CD25 and become highly IL-2 sensitive[56]. CD25 expression level further controls T cell lineage differentiation[57]. Naïve T cells were identified based on the surface expression of CD62L and the absence of CD25. The $CD62L^+$ $CD25^+$ subset includes the activated central memory T cells (Tcm cells) and the $CD62L^-$ $CD25^+$ subset includes the activated effector memory T cells (Tem cells). The $CD62L^-$ $CD25^-$ subset is recognized as resting Tems.

As shown in **Figures 9A&B**, the frequencies of $CD4^+$ and $CD8^+$ T cells in the spleen were 17.6% and 10.9%, respectively. Bone marrow was less abundant in both $CD4^+$ (3.42%) and $CD8^+$ (3.36%) T cells, which is reasonable as bone marrow is not the site for differentiated T cells. $CD4^+$ T cells in the bone marrow, though in a few amounts, showed a certain distribution pattern of subpopulations. $CD4^+$ T cells mainly existed as Tem cells, being either resting (41.1%) or the activated (46.8%). On the other hand, the

activated $CD4^+$ Tem cells only accounted for 9.54% in the spleen. The majority of spleen $CD4^+$ T cells were naïve or resting Tem. Interestingly, most $CD8^+$ T cells in the spleen (86.4%) and bone marrow (60.3%) existed as naïve T cells, and the remaining were resting Tem cells. Finally, the expressions of CD62L and immune checkpoint protein PD-1 in all $CD4^+$ and $CD8^+$ T cells in the bone marrow and spleen followed the similar expression patterns as CD25 in conjugation with CD62L in T cells.

In addition to $CD4^+$ and $CD8^+$ T cells, regulatory T cell marker Foxp3 was used to identify Tregs in the blood and the spleen. The frequencies of Tregs in the blood and spleen were similar, which were 3.49% and 4.67%, respectively (**Figure 10**). Natural Killer cells (NK cells) were characterized by the surface maker NKp46. The frequencies of NK cells were 2.46% in the bone marrow and 1.45% in the spleen, and no difference was observed between the bone marrow and spleen (**Figures 9A&B**).

Overall, our flow cytometric analysis on cellular components in the bone marrow and spleen validated the optimization of our panel design to identify immune phenotype. The accurate location of those cells would be beneficial for our future work on immune cell infiltration.

The expressions of chemokine receptors on the identified immune cells

As indicated previously, CCL2 and CXCL10 were shown to be decreased with the overexpression of Wnt5A in YUMM 1.7 cells. To study the effect of Wnt5A on the migrations of $CCR2^+$ and $CXCR3^+$ immune populations, the expressions of CCR2 and CXCR3 on the identified leukocytes were examined. As shown in **Figures 11A&C**, under normal circumstances, CCR2 was mainly expressed on macrophages and

dendritic cells, with 43.2% of $CCR2^+$ macrophages residing in the spleen and 45.4% of $CCR2^+$ dendritic cells residing in the bone marrow. M-MDSCs, rather than PMN-MDSCs, also expressed significant amounts of CCR2 on the surface, suggesting CCR2 could be associated with monocytic lineages. For lymphocytes, a high proportion of NK cells in the bone marrow (24.4%) and spleen (22.4%) expressed CCR2, which was not observed in $CD4^+$ or $CD8^+$ T cells. Nevertheless, further investigations are needed to understand whether NK cells in the tumor microenvironment migrate in a CCR2-dependent fashion. CXCR3 was expressed on both $CD4^+$ and $CD8^+$ T cells in the bone marrow and spleen (**Figures 11B&D**). Of note, almost all CD8 T cells were CXCR3 positive, while only a subset of CD4 T cells (58.6% in the BM and 43.3% in the spleen) expressed CXCR3.

Furthermore, the expression patterns of CCR2 and CXCR3 in T cells were studied in conjugation with CD62L. The populations of $CD62L^+CD4^+$ and $CD62L^+CD8^+$ T cells were generally CCR2 negative and did not differ significantly between the bone marrow and spleen (**Figure 9**). Although negative for CD62L, $CXCR3^+CD4^+$ T cells accounted for a significant amount of all $CD4^+$ T cells in the bone marrow (44.4%) and spleen (22.9%). Interestingly, 73.5% of $CD8^+$ T cells in the spleen expressed high levels of CD62L and CXCR3. For $CD8^+$ T cells in the bone marrow, while less abundant, the $CD62L^+CXCR3^+$ $CD8^+$ population still accounted for 16.9% (**Figure 9**). Considering that T cells are selected and maturated in the thymus, it is plausible for the difference observed between the BM and spleen.

Overall, $CCR2^+$ granulocytes are mainly macrophages, dendritic cells and M-MDSCs in the bone marrow and spleen. T lymphocytes, except for NK cells, do not typically express CCR2 on the surface. There is a certain amount of non-central memory $CD4^+$ and $CD8^+$ T cells expressing CXCR3 in both the bone marrow and spleen. Finally, almost all $CD8^+$ T cells are CXCR3 positive, with a large majority of $CXCR3^+CD8^+$ central memory T cells enriched in the spleen.

DISCUSSION

Wnt5A signaling has long been implicated as a critical player in melanoma progression. Recent studies suggested that the overexpression of Wnt5A in metastatic melanoma correlates with therapeutic resistance and tumor relapse[13,20,23]. Poorly immunogenic tumors are well known to be negatively associated with the success of immunotherapy. In this study, we have found that cancer-derived Wnt5A specifically reduces chemokines: CCL2 and CXCL10 secretion from YUMM1.7 melanoma cells *in vitro*. Inflammatory cytokines IFN-γ and TNF-α, which play a controversial role in cancer progression, were also downregulated by Wnt5A. Although Wnt5A-overexpressed YUMM1.7 cells also exhibited the enhanced invasive ability based on the deficiency in the selected cytokines and chemokines. We further demonstrated that the tumor-specific infiltration of macrophages and dendritic cells is significantly impeded with the stimulation of rWnt5A. Combined with previous findings on the regulation of MDSCs by Wnt5A in the melanoma microenvironment[24], our work highlights the negative role of Wnt5A in immunomodulation. Finally, our immune profiling analysis provided an optimized method of thoroughly investigating the impact of cancer-derived Wnt5A on the infiltration of leukocytes that influence the TME.

CCL2/CCR2 signaling and Tumor-specific infiltration of Monocytic lineages

As we discussed before, CCL2 level was significantly reduced by Wnt5A in YUMM1.7 cells and its receptor, CCR2, was predominantly expressed on macrophages, dendritic cells and M-MDSCs (**Figure 11**). Out of all chemokines we tested, CCL2 is the

only monocyte attractant protein that is specifically downregulated by Wnt5A. Additionally, based on the evidence of the tumor-specific reductions in macrophage and dendritic cell infiltration (**Figures 6A&B**), we are convinced that melanoma-derived Wnt5A regulates the recruitment of macrophages and dendritic cells to the tumor microenvironment through CCL2/CCR2 signaling. In the future, we aim to build a direct connection between CCL2/CCR2 signaling and the recruitment of macrophages and dendritic cells to the primary tumor by specifically targeting CCL2/CCR2 signaling. Moreover, since CCR2 was also shown to be expressed on NK cells (**Figure 11**), it might be interesting to investigate the infiltration pattern of NK cells in our future study.

In addition to protective roles in cancer, high levels of CCL2 can correlate with adverse prognosis in many cancer patients[58-60]. CCL2/CCR2 signaling has been consistently found to regulate the recruitment of tumor-associated macrophages (M2) and MDSCs[59]. Under normal circumstance, no amount of M2 macrophage was detected in the spleen (**Figure 8**). Future experiments are required to monitor the infiltration of M2 macrophages to the tumor microenvironment. MDSCs are potent immunosuppressive cells and negatively correlate with immunotherapy success in melanoma[61,62]. A recent study indicates that Wnt5A creates an immunosuppressive microenvironment by increasing tumor-specific infiltration of MDSCs[24]. Although a certain proportion of M-MDSCs expressed CCR2 on their surface (**Figure 11**), due to the decreased level of CCL2 ligands as we observed in YUMM cells, site-specific migration of M-MDSCs may not be associated with CCL2/CCR2 signaling. Further

studies on mechanisms behind the Wnt5A-mediated regulation of MDSC are required to fully address the problem.

CXCL10/CXCR3 signaling and lymphocyte recruitment

CXCL10/CXCR3 signaling plays a critical role in attracting immune populations, especially T lymphocytes, into the inflamed sites[34]. Although Wnt5A decreases CXCL10 at both the transcriptional and protein levels, no significant changes were observed in lymphocyte infiltration (**Figures 6E&F**). It is important to note that CXC10 shares the same receptor with CXCL9 and CXCL11, creating redundancy (with CXCL9) in CXCR3 signaling[63]. Since the expressions of CXCL9 and CXCL11 were not specifically regulated by Wnt5A (**Figure 5**), it is reasonable for no effect observed in T cell frequency in the tumor microenvironment with the stimulation of rWnt5A. Furthermore, a previous study shows that in the absence of CXCL10, the frequency of IFN-γ-producing CD8 T lymphocytes is still elevated in a CXCL9-dependent manner[64]. Therefore, due to complex interactions between CXCR3 and its ligands, it is difficult to study the effect on immune infiltration based on a single reduction of CXCL10.

Moreover, the observation of no significant changes in tumor T cells upon the stimulation of rWnt5A (**Figures 6E&F**) leads us to two assumptions. First of all, Wnt5A may not be involved in regulating T cell migration in YUMM1.7 cells. Secondly, YUMM1.7-induced melanoma is poorly immunogenic and may lack tumor-specific T cell infiltration due to the endogenous Wnt5A. Thus, the treatment of rWnt5A did not further reduce the recruitment of T cells. Either way, the comparison of T cell frequencies

between healthy and tumor-bearing mice is required. Since CXCL10 is not an ideal target to study T cell migration, its receptor, CXCR3, can be investigated instead. As shown before, CXCR3 is expressed on a subset of $CD4^+$ T cells and a large majority of memory $CD8^+$ T cells (**Figure 11**). We are interested to see whether the frequencies of these $CXCR3^+$ T cells will be changed within the tumor microenvironment created by YUMM1.7-induced melanoma. If the infiltration of $CXCR3^+$ T cells is impaired in the tumor-bearing group, we wonder whether enhancing CXCR3 signaling would restore tumor T cells and turn the cold into a hot tumor.

The expression of CXCR3 and CCR2 on tumor cells

In addition to the role in recruiting immune cells to restrain melanoma, the expression of CXCR3 on melanoma cells has been shown to promote cancer metastasis[65]. Similarly, CCL2 is demonstrated to promote cancer migration by interacting with CCR2 expressed on tumor cells[66,67]. These studies inspired us to investigate the expressions of CXCR3 and CCR2 on YUMM1.7 cells with the overexpression of Wnt5A. If the levels of CXCR3 and CCR2 are upregulated by Wnt5A, we will further investigate whether these two chemokine receptors would help direct the migration of tumor cells to specific anatomic sites to form metastases. Taken together, a combination of a blockade of chemokine receptors (such as CXCR3 and CCR2) on tumor cells and enhanced chemokine signaling in immune cells may be necessary for tumor regression.

The role of inflammatory cytokines IFN-γ and TNF-α in immunomodulation

The secretion of Inflammatory cytokines IFN-γ and TNF-α is associated with potent anti-tumor effects. The direct impact of TNF-α on immune activation is possibly due to the release of "danger signals" and increases antigen delivery[68]. IFN-γ plays a critical role in both innate and adaptive immunity by synthesizing granzyme B and perforin to initiate apoptosis and upregulating molecules in antigen-presenting machinery: MHC class I and class II[69,70]. IFN-γ also directly regulates the differentiation, activation and development of T lymphocytes[71]. Recent studies add additional sights into the roles of IFN-γ and TNF-α in melanoma, pointing out their contribution to the upregulation of PD-L1[45,46]. Therefore, we wondered whether the immune escape of metastatic melanoma was mediated by IFN-γ and TNF-α. In our results, however, Wnt5A significantly downregulates the expression of IFN-γ and TNF-α, ruling out their direct contribution to the PD-L1-meditated immunosuppression. Therefore, the anti-tumor effects of IFN-γ and TNF-α outweigh the pro-tumorigenic effects in the Wnt5A-overexpressed YUMM cells. We believe that systemic administration or intra-tumoral injection of IFN-γ and TNF-α can restore the anti-tumor immunity against melanoma cells.

In addition to the direct anti-tumor effects of IFN-γ and TNF-α, these two cytokines also have a role in macrophage polarization. Classically activated macrophages (M1) can be induced by IFN-γ alone or combined with LPS or TNF-α, while M2 macrophages was typically regulated by IL-4[72]. Therefore, the decreased levels of IFN-γ and TNF-α might influence M1 polarization. Based on the impaired

tumor infiltration of macrophages due to Wnt5A and decreased level of CCL2 (**Figures 6A&C**), it would greatly impact first-line defense, antigen presentation and T cell activation when M1 polarization is compromised. Moreover, when monocytic lineages are recruited to the tumor, the decreased levels of IFN-γ and TNF-α could lead to an imbalance between M1 and M2 macrophages or an increased level of M2 populations, which would be associated with immunosuppression and tumor progression.

Moreover, IFN-γ is the major driver of the CXCR3 signaling that is associated with the infiltration of the activated T lymphocytes in melanoma[59]. CXCL10 is also inducible by both IFN-γ and TNF-α[59]. Therefore, increasing the levels of IFN-γ and TNF-α in TME can modulate levels of tumor CXCL10 and other CXCR3 ligands, which would further increase intra-tumoral T cell infiltration.

Effects of Matrix Metalloproteinase on chemokines to modulate immune responses

Proteolytic processing of chemokines is a complex process that influences chemotactic activities. Matrix Metalloproteinases (MMPs) have been traditionally associated with the degradation of extracellular matrix (ECM) components, which are important for tumorigenesis, including tumor growth, invasion and metastasis[73,74]. Interestingly, MMPs have been shown to modulate inflammatory and immune responses by processing chemokines[75]. Previous studies demonstrated that MMP-2 and MMP-9 can cleave CCL2 and CXCL10 *in vitro*[75]. The inactivation of CCL2 activity leads to two-fold reduction in monocytic cells migration[75]. In addition, MMP-2 is required

for melanoma invasion and Wnt5A treatment has been shown to increase the levels of MMP-2 in melanoma cells[76]. Therefore, we are interested to know whether Wnt5A can upregulate MMPs, such as MMP-2 and MMP-9, to cleave CCL2 and CXCL10, which would reduce the $CCR2^+$ and $CXCR3^+$ immune populations in the tumor microenvironment.

Combination of Wnt5A inhibition with immune checkpoint blockers

Our observation that links Wnt5A with CCL2 reduction and impaired infiltration of macrophages and dendritic cells suggested the key role of Wnt5A in immunosuppression. The immunosuppressive tumor microenvironment precludes the effect of anti-PD-1 and anti-CTLA-4 therapy. Inhibition of Wnt5A itself or the downstream signals could be an effective way to induce immune responses to suppress the malignant tumor. Holtzhausen et al. showed that inhibition of Wnt signaling synergistically enhances the antitumor effect of anti–CTLA-4 antibody therapy to activate T cells in B16 melanoma[77], suggesting great potentiality of Wnt5A as in combinatorial approaches. In this regard, targeting Wnt5A might turn cold tumors into hot tumors. Therefore, combining Wnt5A inhibition with ICIs can be envisioned as a new therapy to treat metastatic melanoma. More importantly, molecules that can be used to efficiently target Wnt5A signaling pathway are required to be identified.

In summary, our experiments demonstrate that cancer-derived Wnt5A reduces the expressions of chemokines CXCL10 and CCL2 and inflammatory cytokines IFN-γ

and TNF-α in YUMM1.7 cells, exerting an immunomodulatory role in melanoma. The downregulated CCL2/CCR2 signaling impairs tumor-specific infiltration of macrophages and dendritic cells, further establishing Wnt5A as a promising therapeutic target for melanoma. Combining Wnt5A inhibition with immune checkpoint inhibitors would disrupt the immunosuppressive effects and increase the efficacy of immunotherapy. The gating strategy we designed allows us to accurately locate immune cells and is beneficial for our future work on exploring the role of cancer-derived Wnt5A in melanoma immunophenotype regulation.

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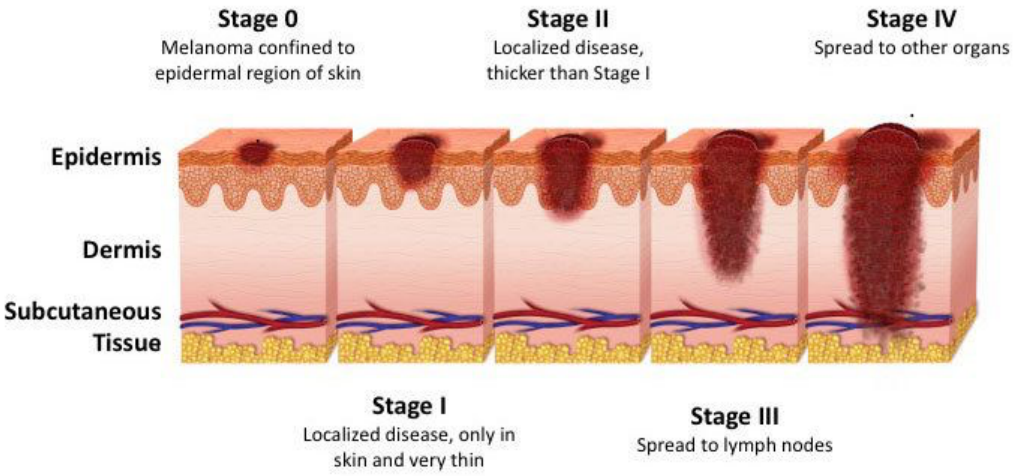

Figure 1 Five Stages of Melanoma.

Stage 0 melanoma is confined to the epidermis. Stage I and stage II melanoma are still localized, but there are cancer cells in both the epidermis and dermis. In stage III melanoma, regional lymph nodes are involved in the spread of cancer cells. Stage IV melanoma has traveled to distant sites. Reprinted from AIM at Melanoma, Retrieved December 10, 2020, from: https://www.aimatmelanoma.org/stages-of-melanoma/

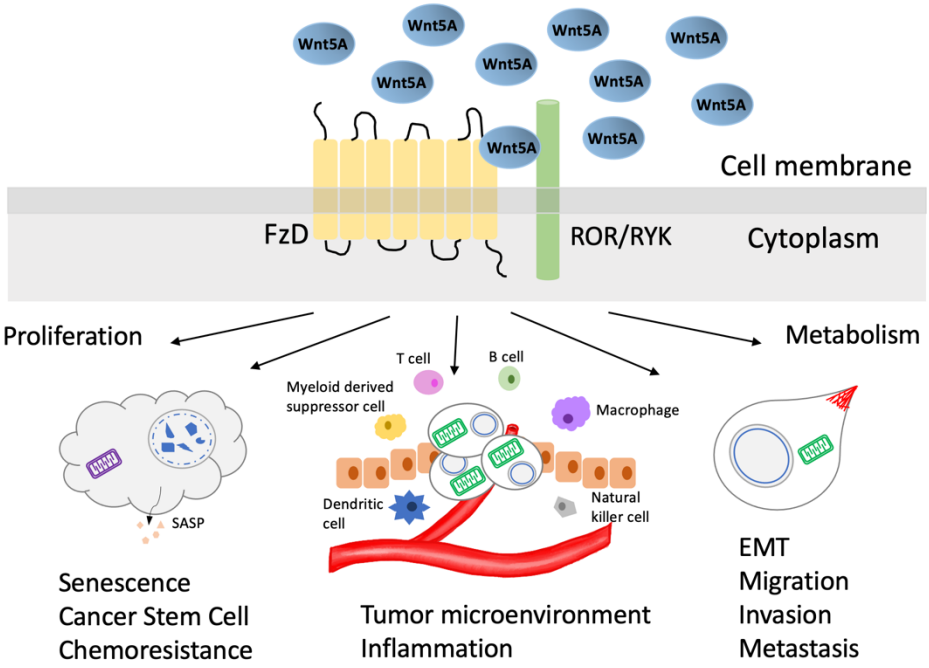

Figure 2 The role of aberrant Wnt5A signaling in cancer progression.

The overexpression of Wnt5A is associated with cancer proliferation, cellular senescence, inflammation, metastasis, metabolism regulation, *etc*. Adapted from Wnt5a Signaling in Cancer, by M. S. Asem, S. Buechler, R. B. Wates, D. L. Miller, & M. S. Stack, 2016, *Cancers* vol 8, 9 79. Copyright 2016 by MDPI.

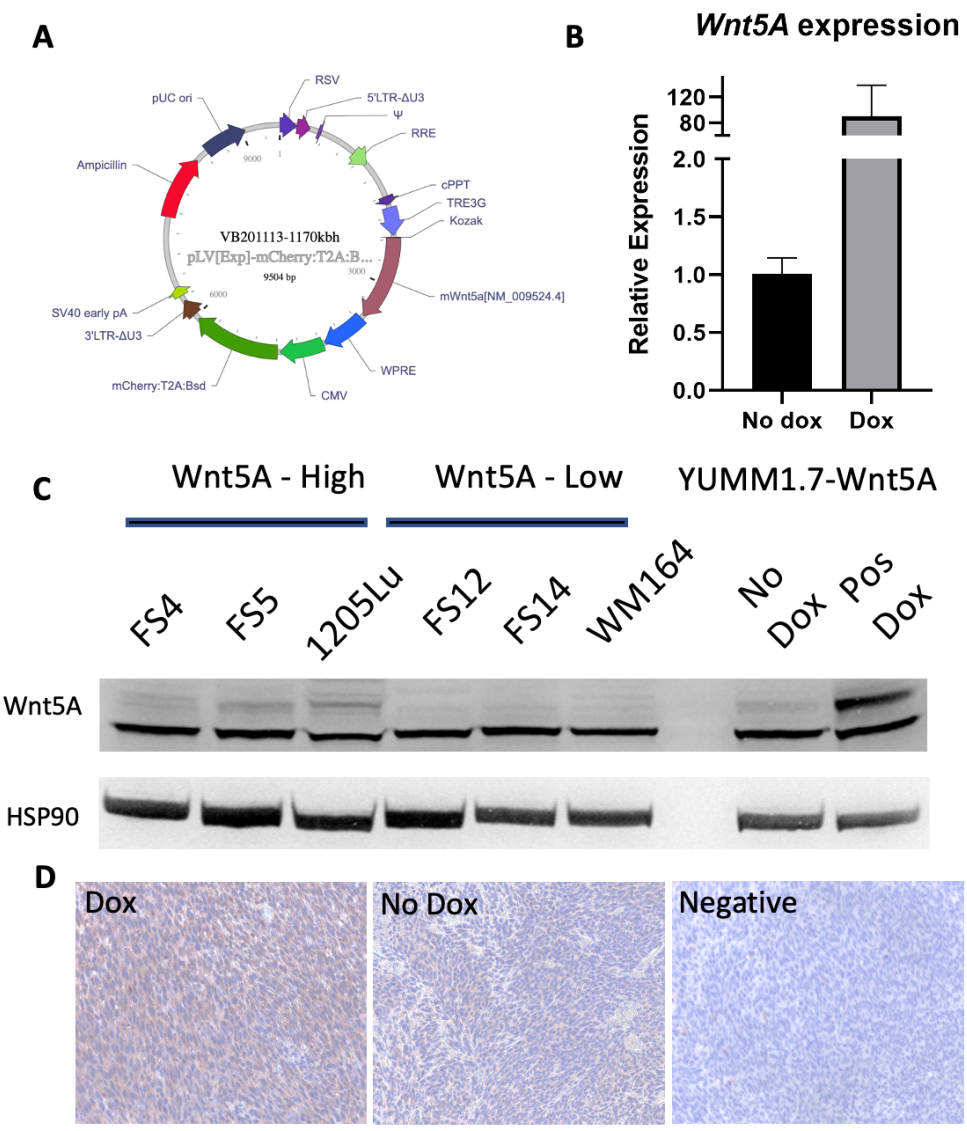

Figure 3 The overexpression of Wnt5A in YUMM1.7 melanoma cells.

(A) Demonstration of vector design for doxycycline-inducible Wnt5A expression. **(B)** RT-PCR analysis show the increased levels of Wnt5A transcripts in YUMM1.7 cells after doxycycline induction. **(C)** The expression levels of Wnt5A in YUMM1.7 melanoma cells before and after Doxycycline induction, as well as in FS4, FS5, 1205Lu, FS12, FS14, WM164 cells which are known for high or low Wnt5A levels. **(D)** Immunohistochemistry assay shows the increased expression of Wnt5A after Dox induction.

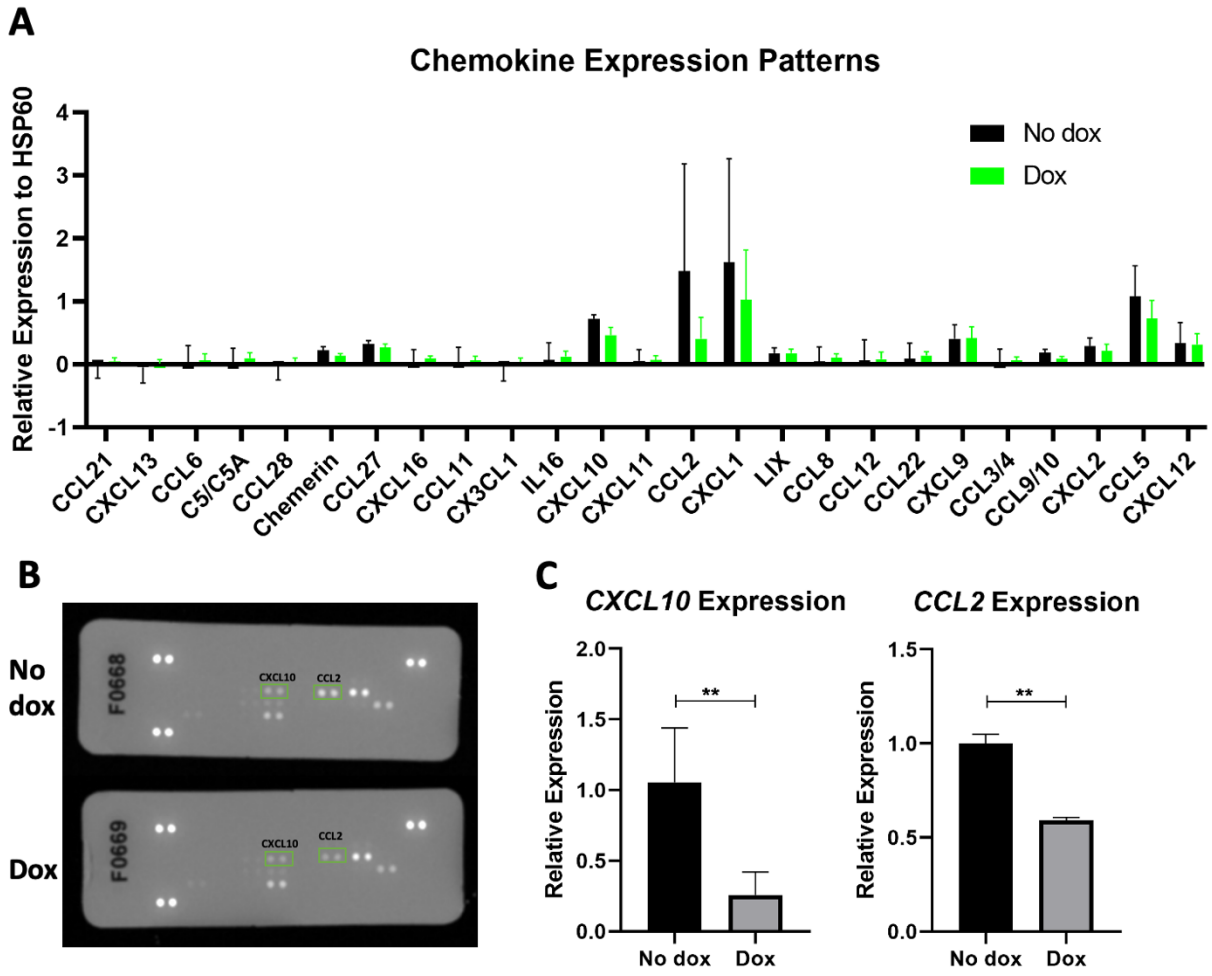

Figure 4 Chemokine expression patterns.

(A) & (B) Mouse chemokine array analysis of 25 chemokines. Among them, CXCL10, CCL2, CXCL1 and CCL5 show the decreased expression levels. **(C)** RT-PCR analysis show the decreased level of CXCL10 and CCL2 transcripts in YUMM1.7 cells after doxycycline induction.

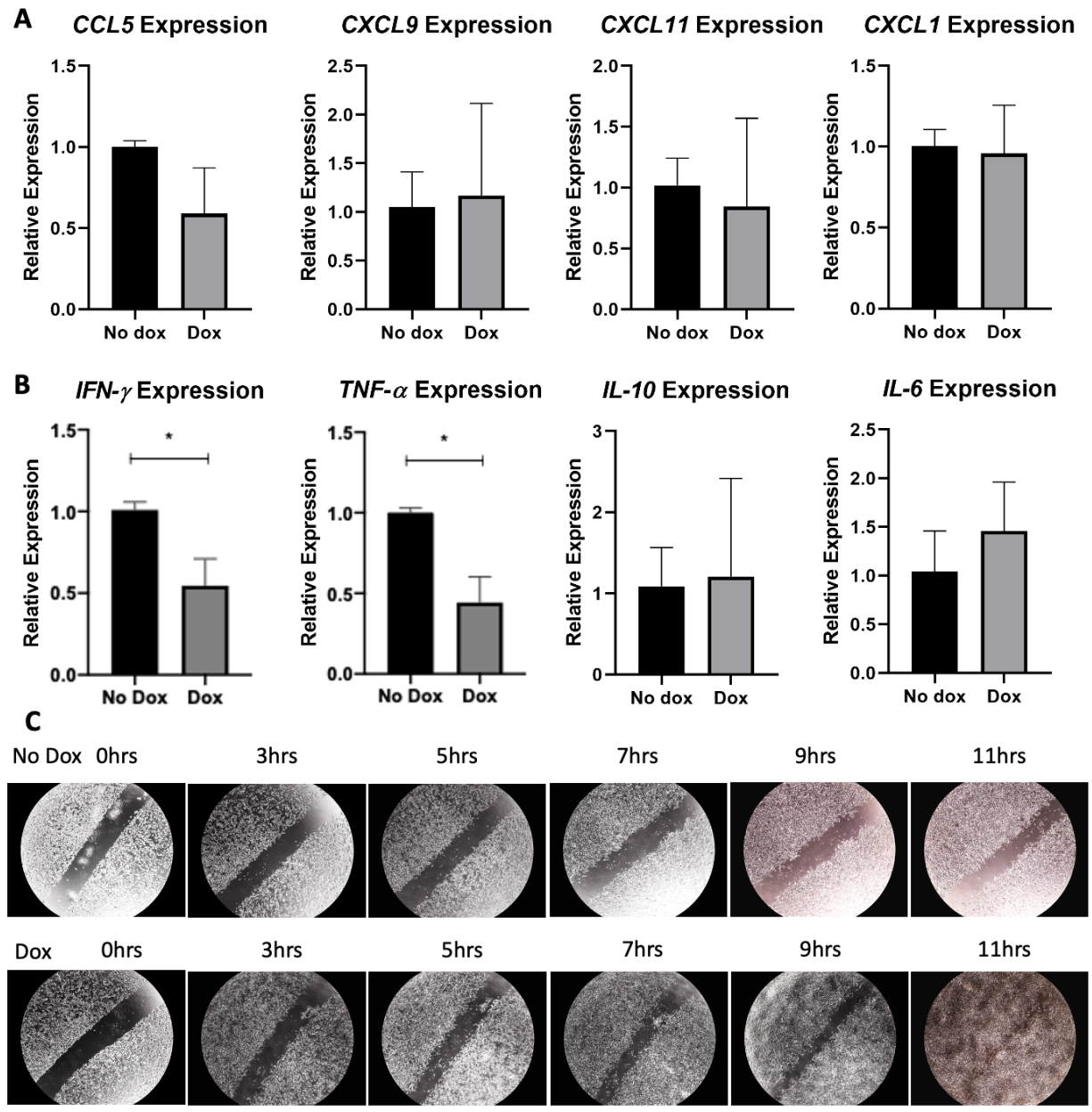

Figure 5 RT-PCR analysis of chemokines and cytokines.

(A) There are no significant changes in the expression of *CCL5*, *CXCL9* and *CXCL11*. (**B**) Pro-inflammatory cytokines *IL-6* and anti-inflammatory cytokine *IL-10* showed no changes in their expression. *IFN-γ* and *TNF-α* were significantly downregulated in the Dox-treated group. (**C**) Wound healing assay of YUMM1.7 cells.

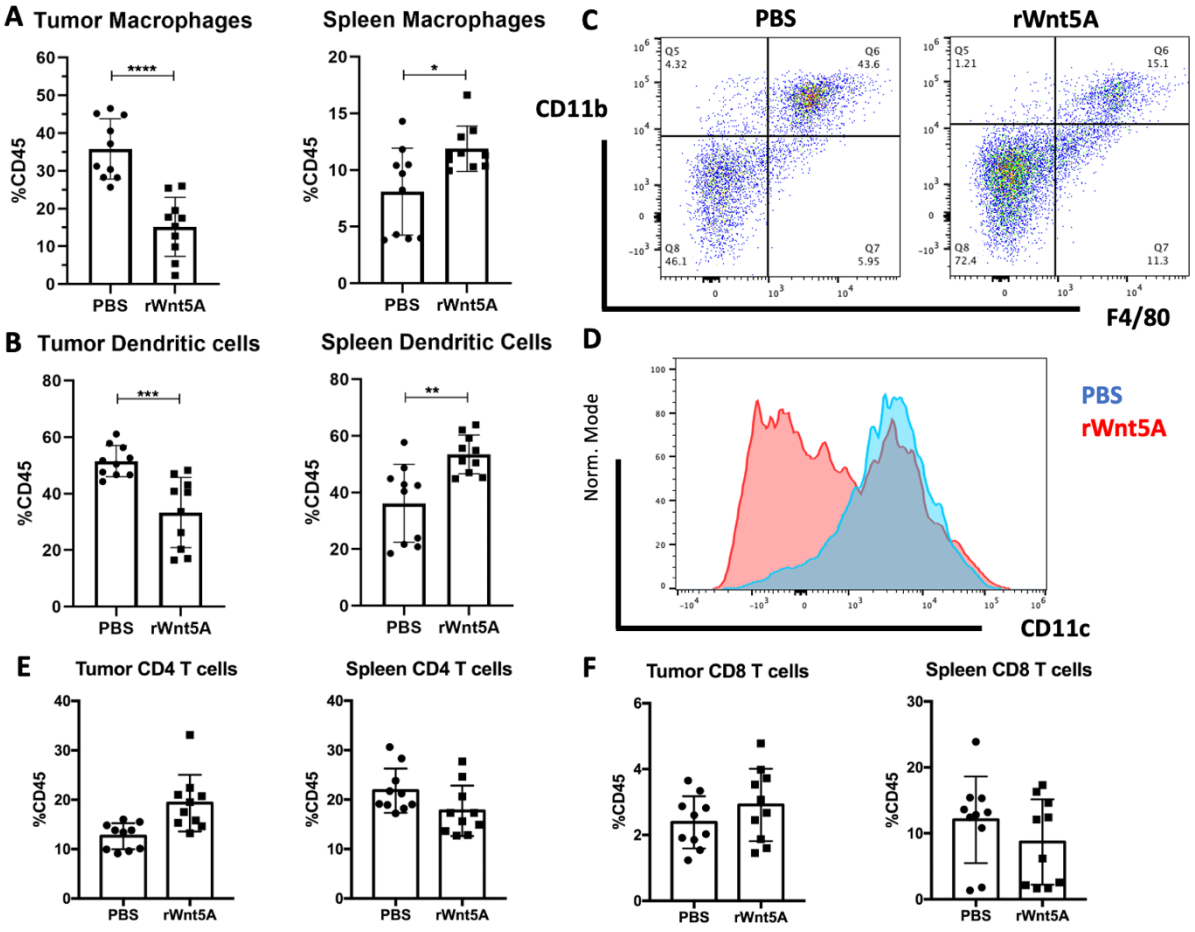

Figure 6 The immune infiltration patterns in vivo with the stimulation of rWnt5A.

(A) & (C) There are significant decreases in Macrophage infiltration to the tumor site with the rWnt5A stimulation. **(B) & (D)** Dendritic cells are less infiltrated into the tumor in the rWnt5A treated group. There are no significant changes in $CD4^+$ T cells (**E**) and $CD8^+$ T cells (**F**) in both the tumor and spleen.

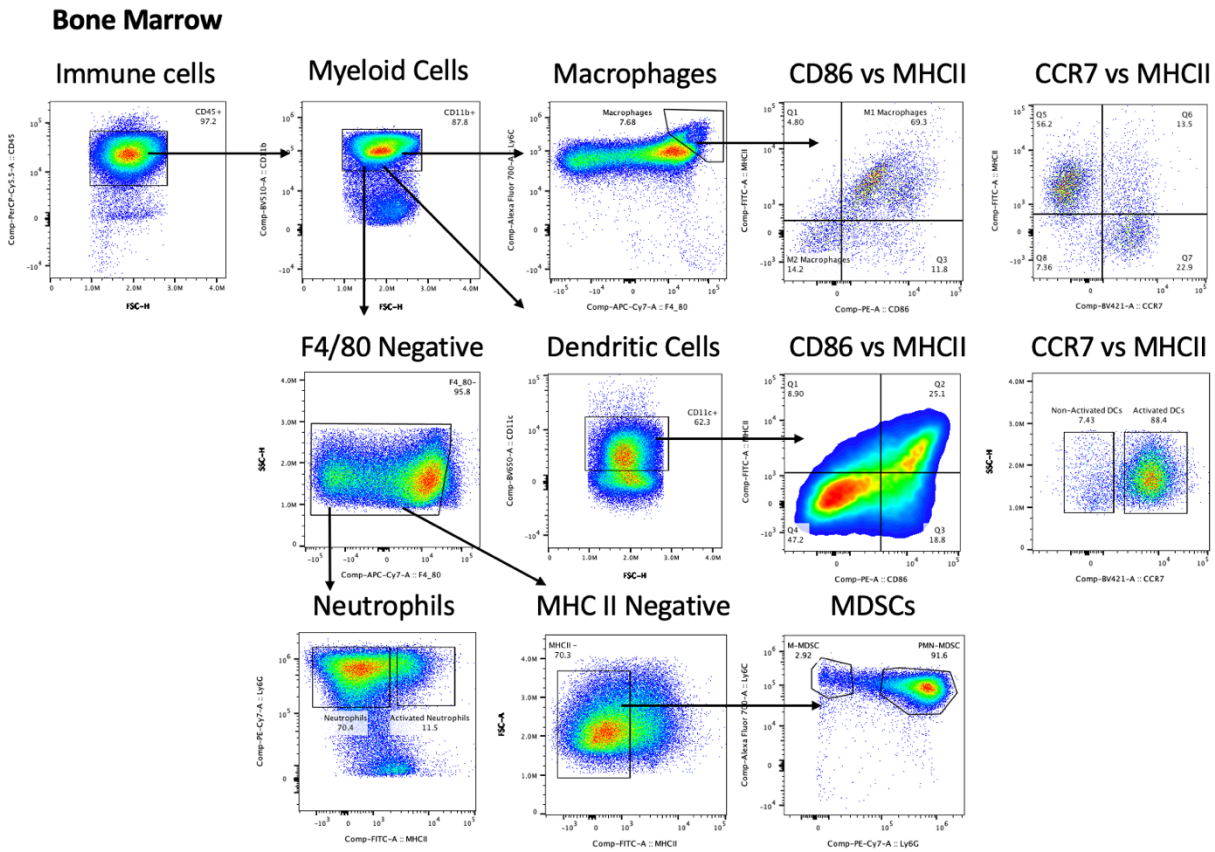

Figure 7 Flow cytometric analysis of myeloid cells in the mice bone marrow.

Density plots of windows and gating strategy used for the identification of myeloid cell populations in the bone marrow. Subsets of macrophages and dendritic cells were identified by MHC class II and co-stimulatory molecule CD86. The migration status was examined by the expression of CCR7.

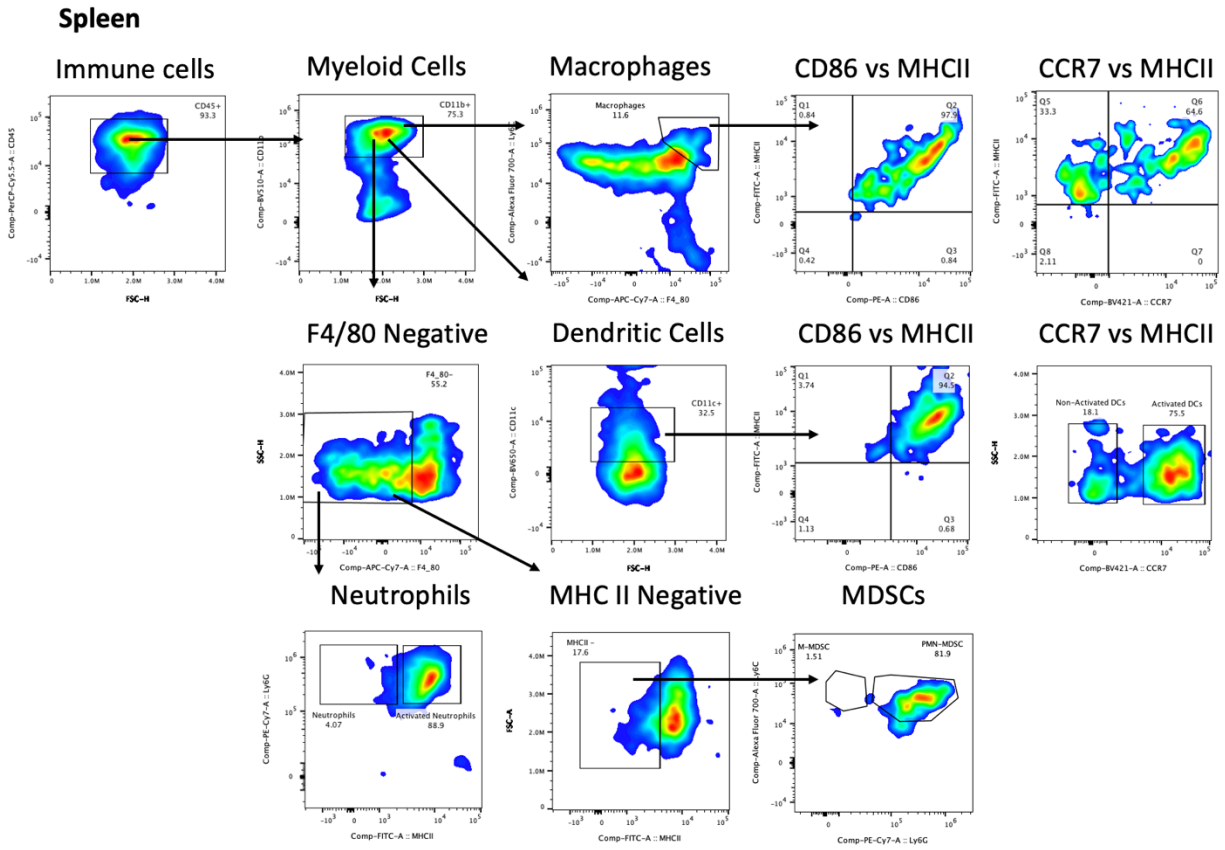

Figure 8 Flow cytometric analysis of myeloid cells in the mice spleen.

Density plots of windows and gating strategy used for the identification of myeloid cell populations in the bone marrow. Subsets of macrophages and dendritic cells were identified by MHC class II and co-stimulatory molecule CD86. The migration status was examined by the expression of CCR7.

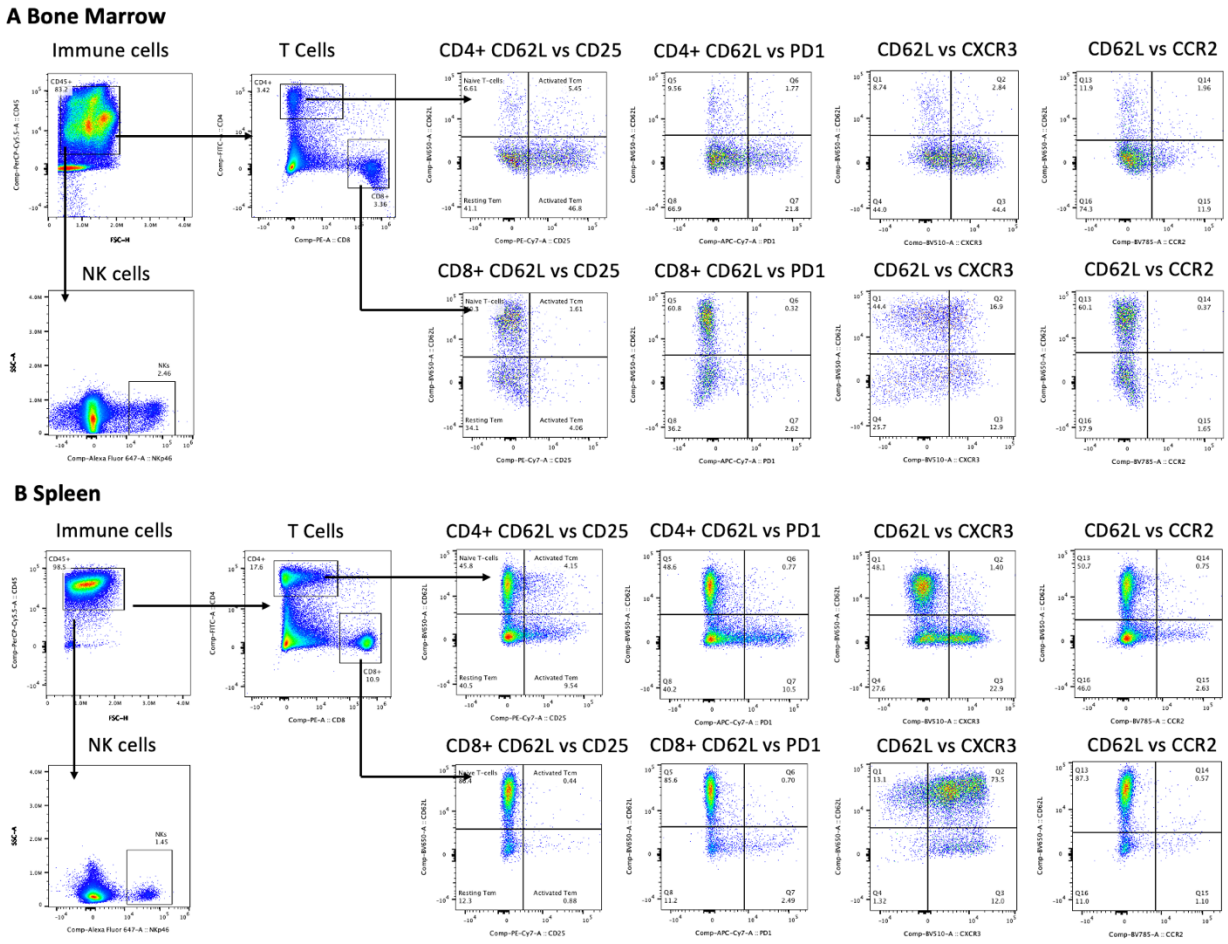

Figure 9 Flow cytometric analysis of lymphoid cells in the mouse bone marrow and spleen.

Density plots of windows and gating strategy used for the identification of immune cell populations in the bone marrow **(A)** and spleen **(B)**.

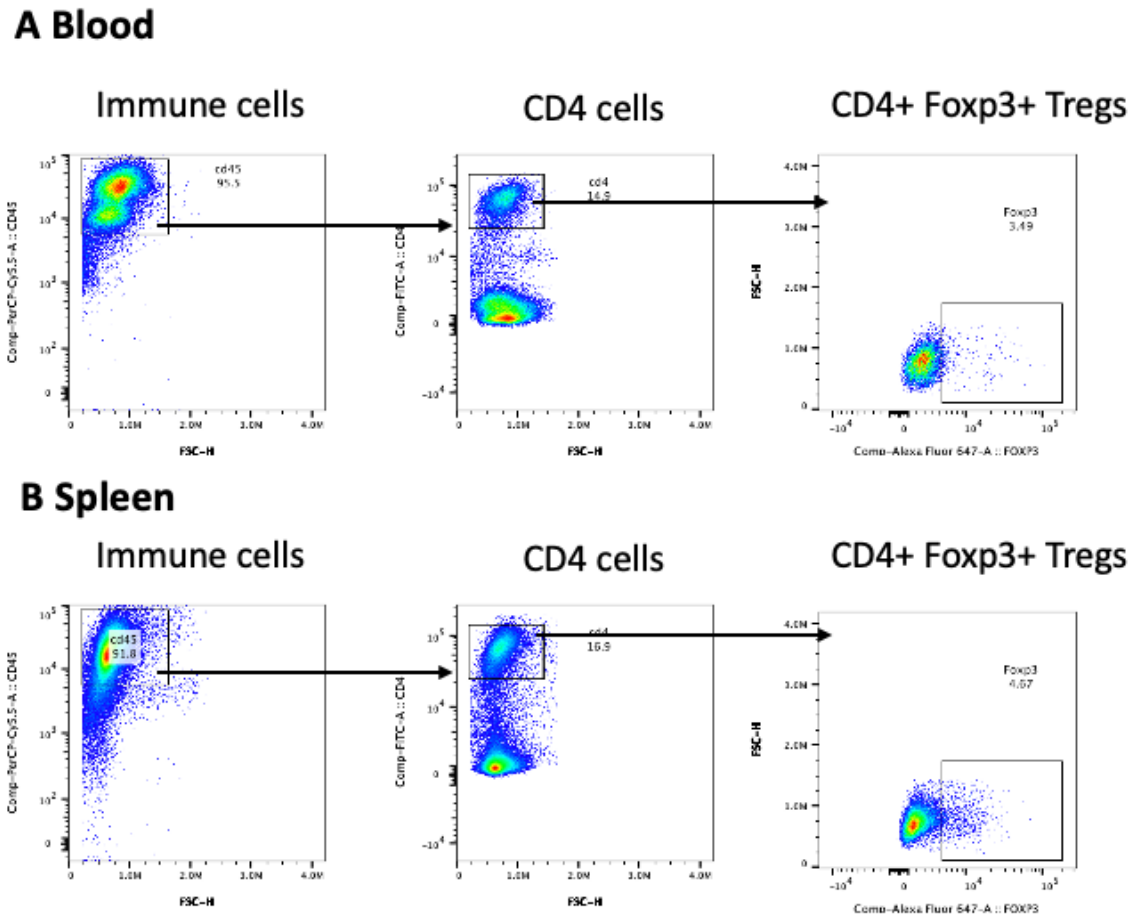

Figure 10 Flow cytometric analysis of Regulatory T cells in the blood and spleen.

Density plots of windows and gating strategy used for the identification of Treg populations in the blood **(A)** and spleen **(B)**.

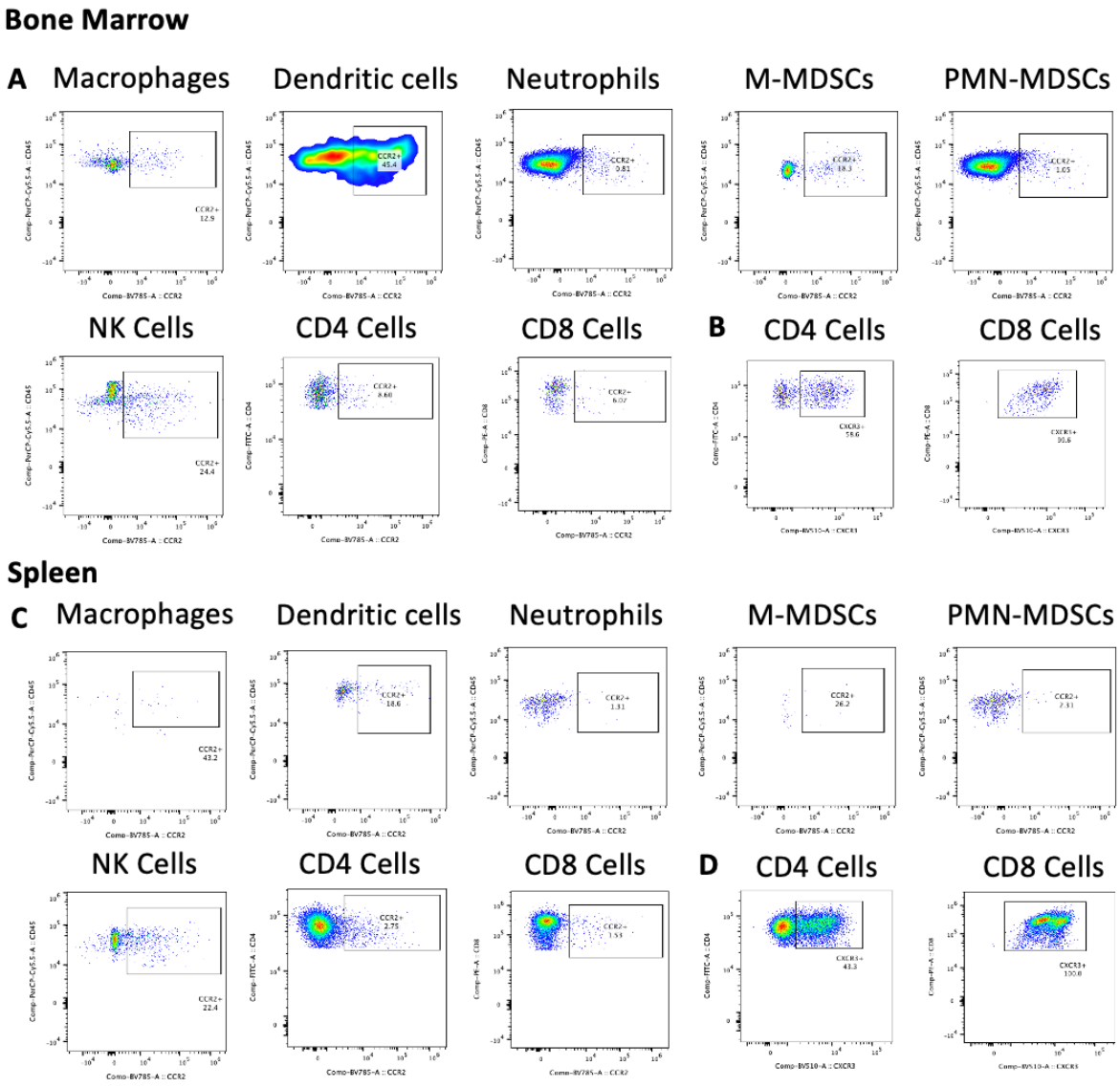

Figure 11 Flow cytometric analysis of chemokine receptor expression on the identified leukocytes.

(A) & (C) CCR2 expression on leukocytes in the bone marrow and spleen. **(B) & (D)** CXCR3 expression on CD4 and CD8 lymphocytes in the bone marrow and spleen.

CURRICULUM VITAE

YIJING GONG

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EDUCATION

ScM in Biochemistry and Molecular Biology (United States) Aug. 2019-Jun. 2021, Johns Hopkins University
Research-based Master Degree

BSc (Honors) in Biological Sciences (China) Aug. 2015-Jul. 2019, Xi'an Jiaotong-Liverpool University
First Class Honorable Degree (Top 10%)
Standardized GPA:3.9/4.0 (WES evaluated)

LABORATORY TECHNIQUES

Biochemistry and Molecular Biology: Gene Cloning, DNA/RNA/Protein isolation, PCR, RT-qPCR, Electrophoresis, Western Blotting, Zymography, Reverse Zymography, Protein Arrays, IHC, IF
Cell biology: Cell culture of melanoma cell lines, Fluorescent Microscopy, *in vivo* animal modelling
Bioinformatics: BLAST, Multiple Sequence Alignments, Phylogenetic Analysis, PyMOL, Snapgene, R programming
Mathematical modeling: Differential Equation model, MATLAB

RESEARCH EXPERIENCE

ScM Student| Thesis Research: ***The role of Wnt5A in melanoma immunophenotype regulation*** Jun. 2020-May 2021
Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health
Supervisors: Prof. Ashani Weeraratna & Dr. Stephen M. Douglass Baltimore, United States

- Induced the overexpression of Wnt5A in YUMM1.7 mouse melanoma cell lines transduced with doxycycline-inducible Wnt5A expression construct.
- Identified chemokine expression patterns in Wnt5A-overexpressed YUMM1.7 cells by chemokine array
- Performed RT-qPCR to screen the expression levels of chemokine transcripts
- Performed the *in vitro* scratch assay to visualize the migration of Wnt5A-overexpressed YUMM1.7 cells
- Performed immunohistochemistry assays to examine the expression levels of several markers in mouse tumor cuts
- Investigated immune infiltrations in C57Bl/6 mice treated with exogenous Wnt5A by flow cytometry
- Performed flow cytometric analysis on the immunophenotype of C57Bl/6 mice
- Investigated the expression levels of chemokine receptors on the identified leukocytes

MHS Student| Thesis Research: ***The aged microenvironment influences tumor progression*** Oct. 2019-May 2020
Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health
Supervisor: Prof. Ashani Weeraratna Baltimore, United States

- Performed literature review on current studies in the aged tumor microenvironment
- Wrote a comprehensive summary detailing how age-induced molecular and cellular changes within stromal fibroblasts, extracellular matrix and the immune microenvironment contribute to tumor progression
- Argued against some current targets and provided new thoughts on cancer therapy

Undergraduate Thesis Researcher| Final Year Project*: A Novel Fibroblast-Matrigel Biocomposite Tailored for MT1-MMP/TACE Inactivation: Impacts on Melanocytes Encapsulation and Inhibition* Sept. 2018-Jun. 2019
Department of Biological Sciences, Xi'an Jiaotong-Liverpool University Suzhou, China
Supervisor: Dr. Meng Huee Lee

- Analyzed the sequestration profiles of the designer TIMPs by reverse zymography
- Characterized the expression levels of Matrix Metalloproteinases (MMPs) in human skin malignant melanoma A375 cells with designer TIMPs by zymography and western blotting
- Identified the expression levels of several extracellular proteins in A375 cells via immunofluorescence microscopy
- Examined the inhibitory effects of baby hamster kidney (BHK) fibroblast with designer TIMPs on A375 cancer cells in the 3D Matrigel

Undergraduate Researcher & Team Leader | Summer Undergraduate Research Fellowship (SURF): ***Detection of Antibiotic Resistance in Bacteria Isolated from Agritainment Farm*** Jun. 2018-Sept. 2018

Department of Biological Sciences, Xi'an Jiaotong-Liverpool University Suzhou, China
Supervisor: Dr. Sekar Raju

- Conducted culture-dependent experiments including Disk Diffusion, Minimal Inhibitory Concentration assay and 48-hour growth curves to reveal the antibiotic resistance of bacteria
- Customized DNA primers to amplify antibiotic-resistant genes via polymerase chain reaction (PCR) and identified them by Agarose Gel Electrophoresis
- Ligated the amplified genes to T vector and performed gene sequencing

Undergraduate Researcher & Team Leader| iGEM competition: *Grenadier Guards – Using Antimicrobial Peptides (AMPs) to Fight Against Staphylococcus aureus* Dec. 2016-Nov. 2017
Department of Biological Sciences, Xi'an Jiaotong-Liverpool University Suzhou, China
Supervisors: Dr. Guoxia Han, Dr. David Chiu & Prof. Dechang Xu

- Designed a genetic circuit in *Lactococcus lactis* against intestinal *Staphylococcus aureus* colonization
  - A sensing device to detect the existence of *S. aureus*
  - AMPs production and lysis of the cell to release AMPs
  - A time-delay device for AMPs' accumulation before lysis
- Established mathematical models to calculate the threshold value for the activation of the sensing device in the genetic circuit and for the efficiency of the time-delay device
- Built a website presenting all ideas and experimental results (http://2017.igem.org/Team:XJTLU-CHINA)
- Delivered a presentation and honored as **Gold Medal Winner** in the competition in Boston, MA, U.S.

AWARDS AND HONORS

Johns Hopkins Master's Tuition (75%) Scholarship Aug. 2019
Student Membership in the Royal Society of Biology Aug. 2019-Present
China National Scholarship (Top 0.05% Nationwide) Oct. 2019
Undergraduate Research Fellowship Aug. 2018
University Academic Excellence Awards (Top 5%) Jul. 2016 & Jul. 2018
International Genetically Engineered Machine (iGEM) competition Gold Medal Winner Nov. 2017

PRESENTATIONS AND CONFERENCES

Professional Presentations

A Novel Fibroblast-Matrigel Biocomposite Tailored for MT1-MMP/TACE Inactivation: Impacts on Melanocytes Encapsulation and Inhibition, **Yijing Gong**, Final Year Project Presentation, XJTLU, China Jun. 2017
Detection of Antibiotic Resistance in Bacteria Isolated from Agritainment Farm, **Yijing Gong**, Jiuhan Shi, Huiting Li, Lei Wang, Summer Undergraduate Research Fellowship (SURF), XJTLU, China Sept. 2018
Grenadier Guard – Using Anti-Microbial Peptides to Fight against Staphylococcus aureus, **Yijing Gong**, Huiting Li, Yuqi Hang, The 2017 iGEM Giant Jamboree, Hynes Convention Center, Boston, MA Nov. 2017

Attended conference

The 17th International Congress of the Society for Melanoma Research Oct. 2020

WORK EXPERIENCE AND EXTRACURRICULAR ACTIVITIES

Department of Learning-Teaching Committee & Student-Staff Liaison Committee Sept. 2017-Jun. 2019
Student Representative Suzhou, China

- Provided feedback on the course design and reported issues raised by students to boost learning processes
- Proposed constructive suggestions: improvement on the online resources and optimization of lab infrastructure

2017 International STEM Science Festival Jul. 2017
Organizer & Volunteer Suzhou, China

- Invited Dr. Jessica Talamas from Cold Spring Harbor Laboratory to deliver a professional talk on scientific research
- Arranged an exhibition and several presentations, introducing the study of synthetic biology to the general public

Wuxi No.2 People's hospital Jul. 2017-Aug. 2017
Clinical laboratory intern Wuxi, China

- Separated the pathogen *Staphylococcus aureus* from patients' samples
- Investigated the efficacy of artificial antimicrobial peptides DRGN-1 by disk diffusion and MBC

Public Speech on XJTLU campus May 2017
Event Organizer & Speaker Suzhou, China

- Invited Dr. Boris Tefsen and Dr. Jia Meng to introduce scientific research and career goals to undergraduates

Presented my project – A new therapy against *Staphylococcus aureus*